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Whole-Genome Analysis of Two-Component Signal Transduction Genes in Fungal Pathogens

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Two-component phosphorelay systems are minimally comprised of a histidine kinase (HK) component, which autophosphorylates in response to an environmental stimulus, and a response regulator (RR) component, which transmits the signal, resulting in an output such as activation of transcription, or of a mitogen-activated protein kinase cascade. The genomes of the yeasts *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, and *Candida albicans* encode one, three, and three HKs, respectively. In contrast, the genome sequences of the filamentous ascomycetes *Neurospora crassa*, *Cochliobolus heterostrophus* (*Bipolaris maydis*), *Gibberella moniliformis* (*Fusarium verticillioides*), and *Botryotinia fuckeliana* (*Botrytis cinerea*) encode an extensive family of two-component signaling proteins. The putative HKs fall into 11 classes. Most of these classes are represented in each filamentous ascomycete species examined. A few of these classes are significantly more prevalent in the fungal pathogens than in the saprobe *N. crassa*, suggesting that these groups contain paralogs required for virulence. Despite the larger numbers of HKs in filamentous ascomycetes than in yeasts, all of the ascomycetes contain virtually the same downstream histidine phosphotransfer proteins and RR proteins, suggesting extensive cross talk or redundancy among HKs.

Two-component histidine kinase (HK) phosphorelay signaling systems are a major mechanism by which some organisms sense and adapt to their environment. These systems have been implicated in regulating diverse processes, including differentiation, chemotaxis, secondary metabolite production, and virulence, in plant and animal pathogens (reviewed in references 24 and 58). In response to an environmental signal, the HK autophosphorylates a conserved histidine residue. This phosphate then is transferred to a conserved aspartic acid residue in a response regulator (RR) protein, resulting in an output such as a change in transcription or regulation of a mitogen-activated protein (MAP) kinase cascade (Fig. 1) (reviewed in references 16 and 57).

HKs have been characterized from bacteria, slime molds, plants, and fungi; however, components of this evolutionarily conserved signaling mechanism have not been identified in animal genome sequences (58). The involvement of HKs in important physiological processes and their absence in animals make two-component signaling pathways attractive potential targets for antimicrobial agents.

Nearly all eukaryotic HKs are hybrids, meaning that both the HK and the RR domains are contained in a single polypeptide (16, 24, 58). Most characterized hybrid HKs, both bacterial and eukaryotic, require an additional phosphorelay step through a histidine phosphotransfer (HPt) domain protein and a second RR protein (16, 58) (Fig. 1). This additional phosphorelay step may allow the organism to integrate multiple input signals into a single output (7).

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Characterization of fungal two-component signaling is relatively limited. Compared to bacteria, yeasts have few HKs (reviewed in reference 47). Like bacteria, many filamentous fungi are significant plant or human pathogens, and analysis of fungal HKs may result in key information about the biology of these pathogens.

The sole *Saccharomyces cerevisiae* HK, ScSln1p, is involved in adaptation to osmotic stress. Under conditions of normal osmolarity, ScSln1p is active, transferring phosphate to the HPt protein ScYpd1p; ScYpd1p relays this phosphate to the RR ScSsk1p (43). Under conditions of high osmolarity, ScSln1p is inactive, and the accumulation of unphosphorylated ScSsk1p activates the *HOG1* MAP kinase cascade, allowing growth under high-osmolarity conditions (31, 43). Likewise, disruption of *ScSLN1* results in the accumulation of unphosphorylated ScSsk1p and thus constitutive activation of the *HOG1* cascade, which is lethal (31).

Schizosaccharomyces pombe has three HK genes, *SpMAK1*, *SpMAK2*, and *SpMAK3* (5, 13). Their encoded hybrid HKs, which do not resemble ScSln1p, except in the HK and RR domains, are implicated in the response to oxidative stress and in cell cycle control (13, 38). *Candida albicans* also has three HK genes, *CaHK1*, *CaNIK1*/*CaCOS1*, and *CaSLN1* (2, 15, 37, 53), all of which are required for normal serum-induced hypha formation and full virulence (59). To date, only a few HK genes from filamentous fungi have been characterized. *Neurospora crassa NcNIK-1*/*OS-1* is implicated in the osmotic response and hyphal development (1, 50). Interestingly, many different mutations in *NcNIK-1*/*OS-1* and related HK genes in other fungal species result in resistance to certain fungicides (17, 33, 40), possibly because these fungicides affect downstream signaling pathways. Indeed, wild-type genes corresponding to other osmosensitive, fungicide-resistant *N. crassa* mutants recently were identified as components of the *HOG1* MAP kinase pathway (21, 61). *Aspergillus nidulans AnTcsA*

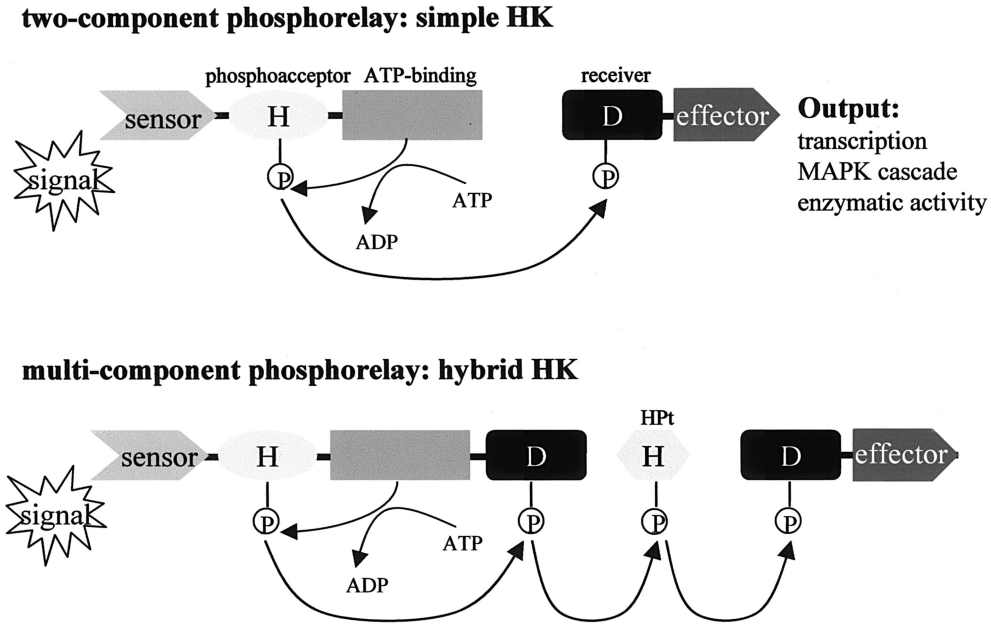

FIG. 1. General schematic diagram of phosphorelay signaling (adapted from reference 57). An external signal is sensed by the sensor domain and triggers phosphorylation of a conserved histidine residue (H). This phosphate is transferred to a conserved aspartic acid residue (D) in the RR receiver domain, activating the effector domain to result in an output such as activation of transcription or a MAP kinase (MAPK) cascade. For a simple two-component system (top), the HK (sensor, phosphoacceptor, and ATP-binding domains) and the RR are separate proteins. A hybrid HK protein (bottom) contains both HK and RR domains and generally requires additional rounds of phosphorelay through an HPt domain and a second RR protein. The HPt domain can be part of the hybrid HK protein or a separate protein. Most eukaryotic and all fungal HKs are hybrids.

(two-component signaling) and its apparent *Aspergillus fumigatus* ortholog, *AfFOS-1*, may regulate the formation of asexual spores (conidia) (8, 44). An *SLN1*-related HK gene in *A. nidulans*, *AnTcsB*, was reported recently, but an analysis of a deletion mutant did not yield any obvious clues to its function (22).

These few HK genes in filamentous fungi have been identified primarily through complementation of a mutant allele (50), degenerate PCR (1, 8, 44), or cDNA library sequences (22). In contrast, whole-genome sequencing offers the opportunity to identify all of the HK genes in a given organism. The recently sequenced model filamentous fungus, *N. crassa*, contains 11 putative HK genes (23). Thus, filamentous ascomycetes likely contain significantly larger numbers of HKs than yeasts, perhaps reflecting the greater range of environmental niches that these fungi occupy.

Life on a host is a highly specialized environmental niche. Most ascomycete plant pathogens are concentrated in three classes of euascomycetes, *Dothideomycetes* (loculoascomycetes), *Leotiomycetes* (inoperculate discomycetes), and *Sordariomycetes* (pyrenomycetes) (11). We identified two-component signaling proteins from a representative of each class through analysis of high-coverage shotgun genome sequences. The taxa included *Cochliobolus heterostrophus* (a *Dothideomycete*; anamorph, *Bipolaris maydis*), the cause of southern corn leaf blight; *Botryotinia fuckeliana* (a *Leotiomycete*; anamorph, *Botrytis cinerea*), a necrotrophic pathogen causing gray mold on a broad range of hosts (45); and *Gibberella moniliformis* (a *Sordariomycete*; anamorph, *Fusarium verticillioides*), a pathogen of maize that produces secondary metabolites that are extremely toxic to mammals, making it a considerable agricultural concern (19). The saprophyte *N. crassa* is also a *Sordariomycete*. Here, we report the whole-genome analysis of two-component signaling genes from each of these diverse filamentous ascomycete plant pathogens and a comparison of these genes with those identified in *N. crassa* and yeasts.

MATERIALS AND METHODS

Genome assembly information. *N. crassa* DNA and protein sequence predictions (assembly 3) were obtained from the *Neurospora* Sequencing Project, Whitehead Institute/MIT Center for Genome Research (WICGR) (http://www-genome.wi.mit.edu). Preliminary sequence data for *A. fumigatus* were queried at The Institute for Genomic Research (TIGR) website (http://www.tigr.org). Shotgun sequence assemblies (Torrey Mesa Research Institute [TMRI]/Syngenta) for *B. fuckeliana* strain B05.10 (~5-fold coverage), *C. heterostrophus* strain C4 (ATCC 48331) (~5-fold), *G. moniliformis* strain FGSC 7600 (ATCC 38932) (~5-fold), and *Gibberella zeae* strain Z3639 (~2-fold) were used for these studies.

Gene identification. Fungal HK genes were identified through a combination of approaches. BLAST searches (3) were performed against TMRI fungal genome sequence databases. Consensus Protein Families Database (Pfam; www.sanger.ac.uk/software/Pfam/) sequences for the HK phosphoacceptor (PFAM00512) and RR (PFAM00072) were used in TBLASTN searches of each fungal genome. In addition, a set of computer-generated gene predictions analyzed for Pfam domains (generated by Darrell Ricke, Bioinformatics, TMRI) was parsed to identify potential genes encoding the above domains. Once annotated, putative HK amino acid sequences were compared (TBLASTN) against each fungal genome as a means to identify HKs not found by other methods. This method would identify HKs whose conserved HK and RR domains might be missing due to gaps in the genome sequence. All approaches yielded a similar set of genes. RR genes were identified in a similar manner. HPt genes were identified by using *ScYPD1* and *SpMPR1* for BLAST searches.

Sequence annotation. Putative amino acid sequences were determined through manual annotation by using both consensus intron splice sequences (26) and regions of homology among fungal HKs identified through TBLASTX and

TBLASTN analyses. Thus, coding sequence predictions for highly conserved genes likely are more accurate than those for more divergent HK genes. Likewise, intron predictions for the conserved C-terminal regions shared by all HKs should be more accurate than predictions for N-terminal introns and the translation start site. In some cases, particularly for *B. fuckeliana* HK genes, perhaps due to sequence quality issues, we were unable to make reasonable intron predictions for the entire gene and instead made predictions for each conserved Pfam domain, in order to use these genes in alignments. Note that, while in general, the WICGR automated annotation was used for *N. crassa* amino acid sequences, in two cases (NCU05790.1 and NCU09520.1), the alignments suggested that manual reannotation, as described above for the TMRI/Syngenta sequences, would allow more accurate analysis. These sequences are hereafter referred to as NCU05790 and NCU09520, the WICGR version numbers having been dropped.

Conserved Pfam domains were identified by using the DeCypher hidden Markov model (protein sequence versus hidden Markov model) search algorithm (TimeLogic, Crystal Bay, Nev.). For the alignments used to generate Fig. 2, domain boundaries were refined as needed based on initial alignments. For domain structure drawings, full-length alignments were made for each class of HKs, and conserved domain positions were compared among members of the class.

Sequence alignment and trees. Sequence alignments were performed by using either ClustalW (55) or T-Coffee (39). Slight manual adjustments were made as deemed appropriate by visual inspection of the alignments. Phylograms were made by using parsimony in PAUP4.0b8 (Sinauer Associates, Sunderland, Mass.) with a minimum of 100 stepwise additions. Gaps were treated as a 21st amino acid.

For the HK phylogram (Fig. 2A), the ClustalW alignment was 584 characters: 7 constant, 122 variable uninformative, and 455 parsimony informative. Bootstrapping involved 1,000 repetitions (10 stepwise additions each). For the HHK1 phylogram (see Fig. 4), the T-Coffee alignment was 3,145 characters: 93 constant, 2,194 uninformative, and 858 parsimony informative.

Nucleotide sequence accession numbers. The *C. heterostrophus* (accession no. AY456004 to AY456029), *G. moniliformis* (accession no. AY456030 to AY456050), and *B. fuckeliana* (accession no. AY456051 to AY456072) two-component signaling molecule sequences and two reannotated *N. crassa* sequences (accession no. AY456073 [NCU05790] and AY456074 [NCU09520]) have been deposited in GenBank.

RESULTS AND DISCUSSION

Identification and comparative analysis of fungal HK genes. Putative HK genes were identified by the criteria that they should encode both the conserved phosphoacceptor (PFAM00512) and the ATP-binding (PFAM02518) domains (Fig. 1). We identified 21 HK genes in *C. heterostrophus*, 20 in *B. fuckeliana*, and 16 in *G. moniliformis* (Table 1 and Fig. 2A). As described above, the WICGR *N. crassa* (assembly 3) gene predictions include 11 HK genes (23). Like almost all described eukaryotic HKs, all fungal HKs identified were hybrids, containing both HK and RR domains (PFAM00072). Only one putative HK, BfPHY3, appears to be missing the conserved phosphoaccepting histidine (Fig. 3).

TBLASTN analysis of a lower-coverage TMRI/Syngenta shotgun sequence of *G. zeae* (anamorph, *F. graminearum*), which causes head blight (scab) of wheat, corn, and barley (19), revealed that each of the 16 *G. moniliformis* HK genes had a clear counterpart in *G. zeae*, with the exception of the *GmTCS1* gene (N. L. Catlett, unpublished data). Initially, we thought that this omission might have been due to imperfect sequence coverage, as the *GmTCS1* gene product is relatively small (685 amino acids, no introns) and all other *G. zeae* HK gene sequences are incomplete, with most spread across multiple sequence contigs. However, the recently released Whitehead Institute *G. zeae* genome sequence (10-fold coverage; *Fusarium graminearum* sequencing project website [www=genome.wi.mit.edu]) also lacks a *GmTCS1* ortholog.

Many of these HKs contain previously described domains, including PAS/PAC domains (54), GAF domains (PFAM01590), and protein kinase domains (PFAM00069), N terminal to the conserved HK and RR domains. Both GAF and PAS domains are highly divergent, versatile ligand-binding domains. GAF domains bind cyclic GMP and chromophores (10). PAS (Per-ARNT-Sim) domains are frequently followed by a 40- to 45-amino-acid PAC motif (41). PAS and PAC domains are proposed to comprise a single structural element (54) and thus are referred to as PAS/PAC domains here. PAS/PAC domains sense redox potential, cellular oxygen, cellular energy, and light and are found in proteins regulating circadian rhythms and hypoxia responses (transcription factors and ion channels) as well as in input domains for two-component signaling systems (reviewed in reference 54). PAS/PAC and GAF domains are widely distributed but are found primarily in proteins involved in signaling or regulation of transcription (4).

Phylogenetic relationships among HKs were inferred from alignments of the conserved HK (PFAM00512 and PFAM02518) and RR (PFAM00072) regions of the predicted proteins, as these regions were conserved among all fungal HKs. The three *C. albicans* HKs were included in this analysis because CaSLN1 and CaNIK1 grouped with HKs from the filamentous ascomycetes. The three *S. pombe* sequences were omitted because, in preliminary analyses, these sequences did not group with other ascomycete HK sequences. Phylogenetic analysis was also done with single alignments of each of the three conserved PFAM domains (data not shown). This analysis resulted in the same major groupings of HKs as those identified by concatenated alignments of all three domains, suggesting that high similarity in one domain correlates with high similarity throughout the length of the protein. This grouping of fungal HKs was also confirmed by using TBLASTX and TBLASTN searches of fungal genome sequences to identify which HK from each species was the "best hit" for a given HK. The N-terminal regions of the HKs were conserved within a group, and each group contained a distinct N-terminal region.

This phylogenetic analysis revealed 11 major groups of euascomycete (*C. heterostrophus*, *G. moniliformis*, *N. crassa*, and *B. fuckeliana*) HKs (Fig. 2 and Table 2). Many of these groups contain HKs that are highly conserved in filamentous ascomycetes. Other groups are more divergent, containing gene families that have expanded within species and few clear orthologs between species. These groupings suggest that some HK genes are necessary for basic functions shared by most or all ascomycetes (e.g., osmosensing), while others may have evolved to adapt to specific aspects of the lifestyle of a pathogen. In contrast, structural genes, such as those for kinesin-like motor proteins, have a nearly one-to-one correspondence of orthologs across ascomycete genomes (49), and genes involved in secondary metabolite synthesis, such as polyketide synthase genes, have relatively few orthologous pairs shared between genomes, even between the two *Gibberella* genomes (27a).

Common orthologs. Six HK groups (III, V, VI, VIII, IX, and X) contain closely related sequences from each euascomycete species examined (Fig. 2 and Table 2). Thus, the sequences within each group may represent orthologs with common functions. These apparent orthologs were given the same gene name and are referred to here with a two-letter prefix to

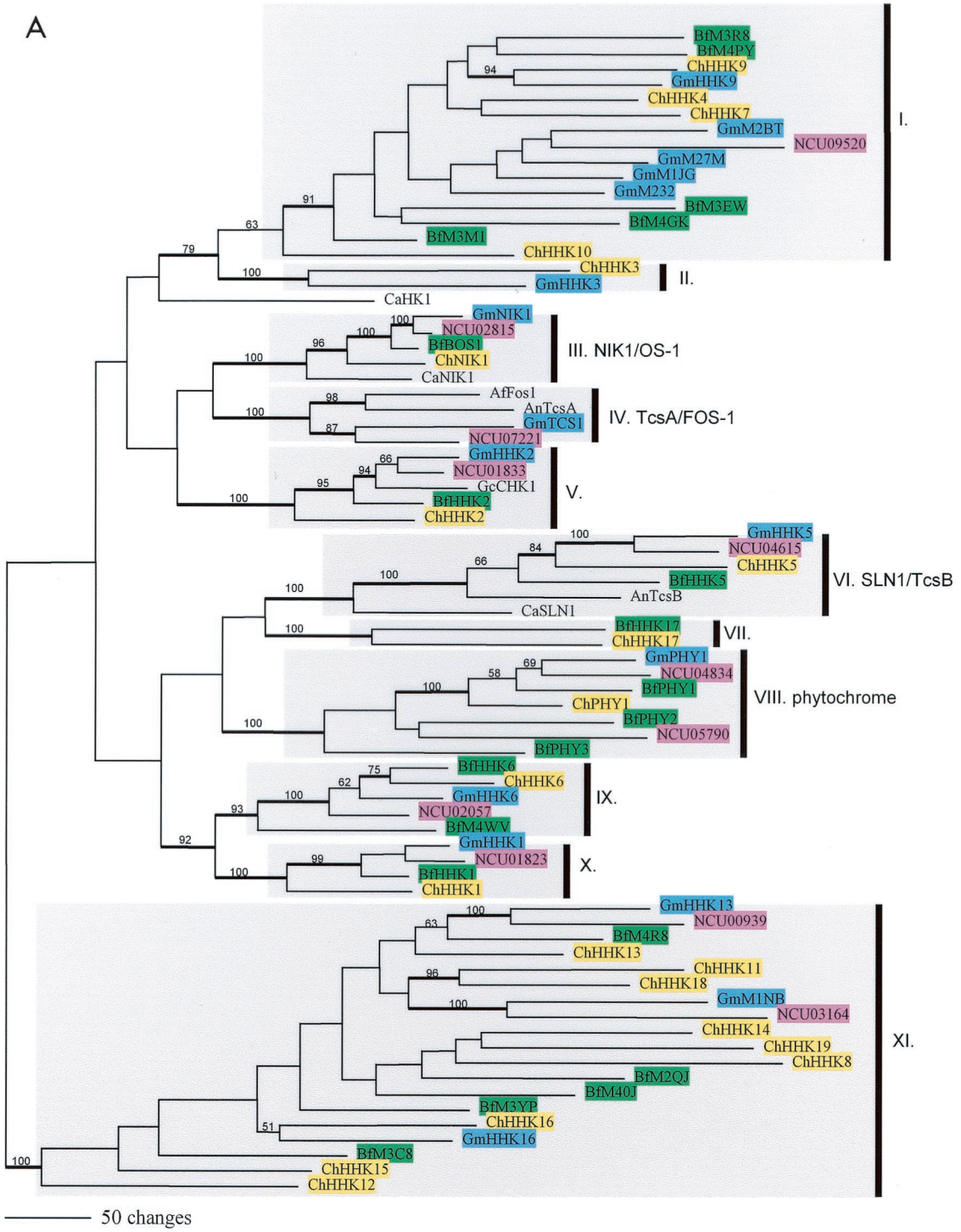

FIG. 2. Midpoint-rooted phylogram of fungal HKs. (A) Conserved phosphoacceptor (PFAM00512), ATP-binding (PFAM02518), and RR receiver (PFAM00072) domain amino acid sequences were aligned by using ClustalW. The phylogram was constructed by using parsimony (PAUP4.0b8). One of the two trees obtained is shown. *N. crassa* predicted protein sequences (pink) are identified by WICGR unique identifier numbers. *C. heterostrophus* (yellow, Ch), *G. moniliformis* (blue, Gm), and *B. fuckeliana* (green, Bf) sequences were obtained by homology and splice consensus-based manual predictions from TMRI fungal genome sequences. Other protein sequences are from GenBank. Percent confidence obtained by bootstrap analysis (1,000 repetitions, 10 stepwise additions each) is shown for branches with greater than 50% support. (B) Scaled cartoon of domain structure for a representative protein from each group. For group XI, some members have additional PAS/PAC domains or a less conserved GAF domain.

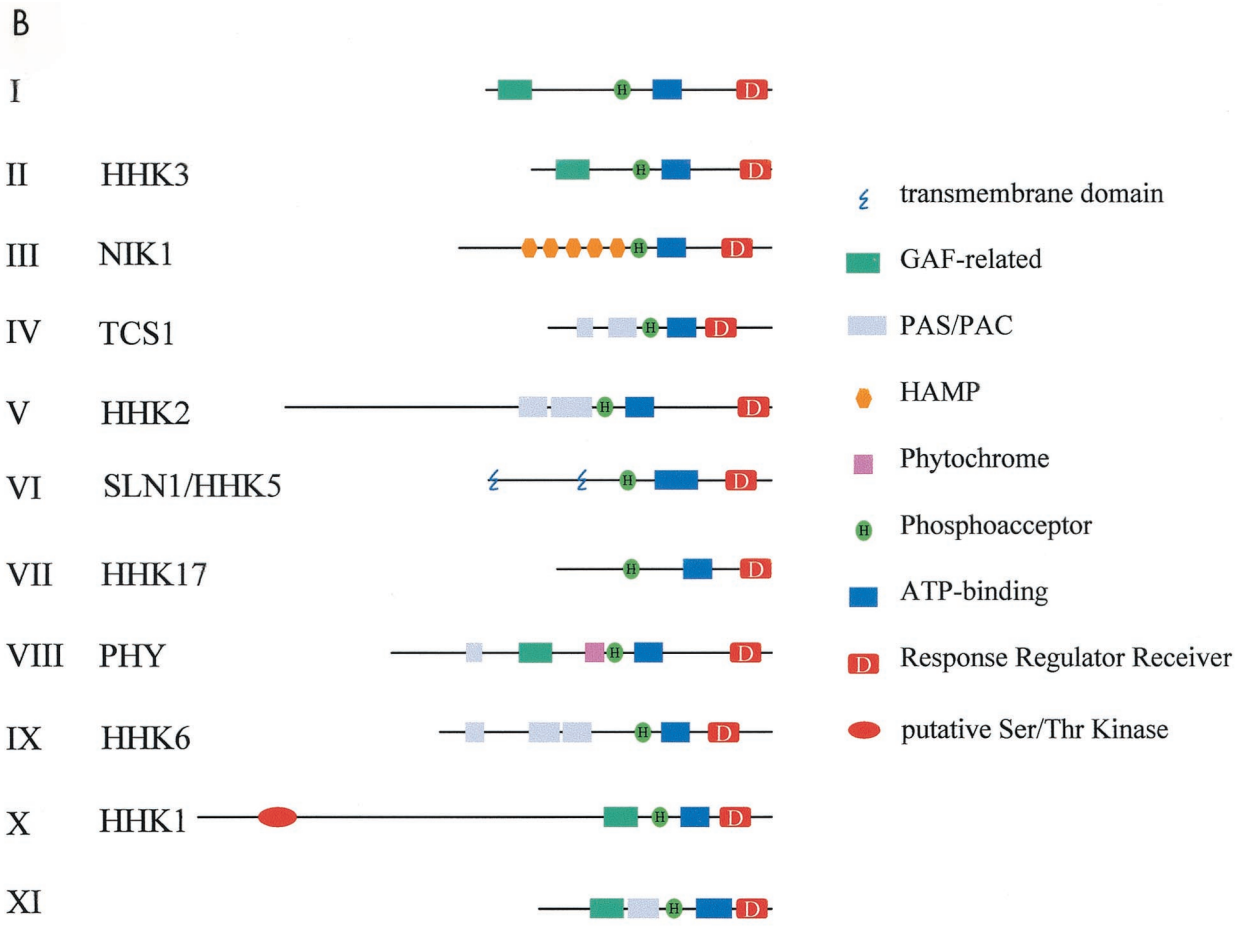

FIG. 2—*Continued.*

designate the genus and species (i.e., *Ch* for *C. heterostrophus*, Gm for *G. moniliformis*, and *Bf* for *B. fuckeliana*). They include *NIK1* (group III), *HHK1* (group X), *HHK2* (group V), *HHK5* (group VI), *PHY1* (group VIII), and *HHK6* (group IX). These genes may represent the core set of HK genes for most filamentous euascomycetes.

Filamentous ascomycetes fall in subphylum *Pezizomycotina* (*Euascomycetes*), which comprises the majority of known ascomycete species. *S. pombe* falls in subphylum *Taphrinomycotina* (*Archiascomycetes*), and *S. cerevisiae* and *C. albicans* fall in subphylum *Sacchromycotina* (*Hemiascomycetes*). Consequently, there is a publicly available high-quality genome sequence for at least one representative of each ascomycete subphylum. N-terminal domain structure analysis, in addition to the phylogenetic analysis based only on domains conserved among all hybrid HKs, suggested that four of the most conserved euascomycete HKs (NIK1, HHK5 [AnTcsB], HHK1, and HHK2) have counterparts in at least one of the yeasts (Fig. 2 and 4). Thus, an ancestral ascomycete likely possessed HKs similar to each. Despite this cross-species conservation, very little is known about the signals perceived by and functions of these HKs.

TABLE 1. Two-component signaling genes in fungal and other genomes

Genome	No. of genes for:				Reference
	HK		HPt	RR[a]	
	Simple (HK alone)	Hybrid (HK + RR)			
Escherichia coli	23	5	5[b]	32	35
Arabidopsis thaliana[c]	1	7	5	22	48
Saccharomyces cerevisiae	0	1	1	2	47
Schizosaccharomyces pombe	0	3	1	2	47
Gibberella moniliformis	0	16	1	3	
Cochliobolus heterostrophus	0	21	1	3	
Botryotinia fuckeliana	0	20	1	2	
Neurospora crassa	0	11	1	2	

[a] Not counting the *RIM15* ortholog.
[b] Four *E. coli* HPt domains are part of a hybrid kinase.
[c] Not including divergent sequences.

The putative osmosensor NIK1 was highly conserved among the euascomycetes. Orthologs of this HK have been reported for many ascomycete species, including *C. albicans*, *N. crassa*, and *B. fuckeliana* (1, 2, 17, 37, 53). NIK1 orthologs have a unique N-terminal region consisting of HAMP domain repeats (PFAM000672) (Fig. 2B). HAMP domains are found in signaling-related proteins, including HKs, adenylyl cyclases, methyl-accepting chemotaxis proteins, and phosphatases (9). The precise function of these domains is unknown; however, mutations in the NIK1 HAMP repeat region are responsible

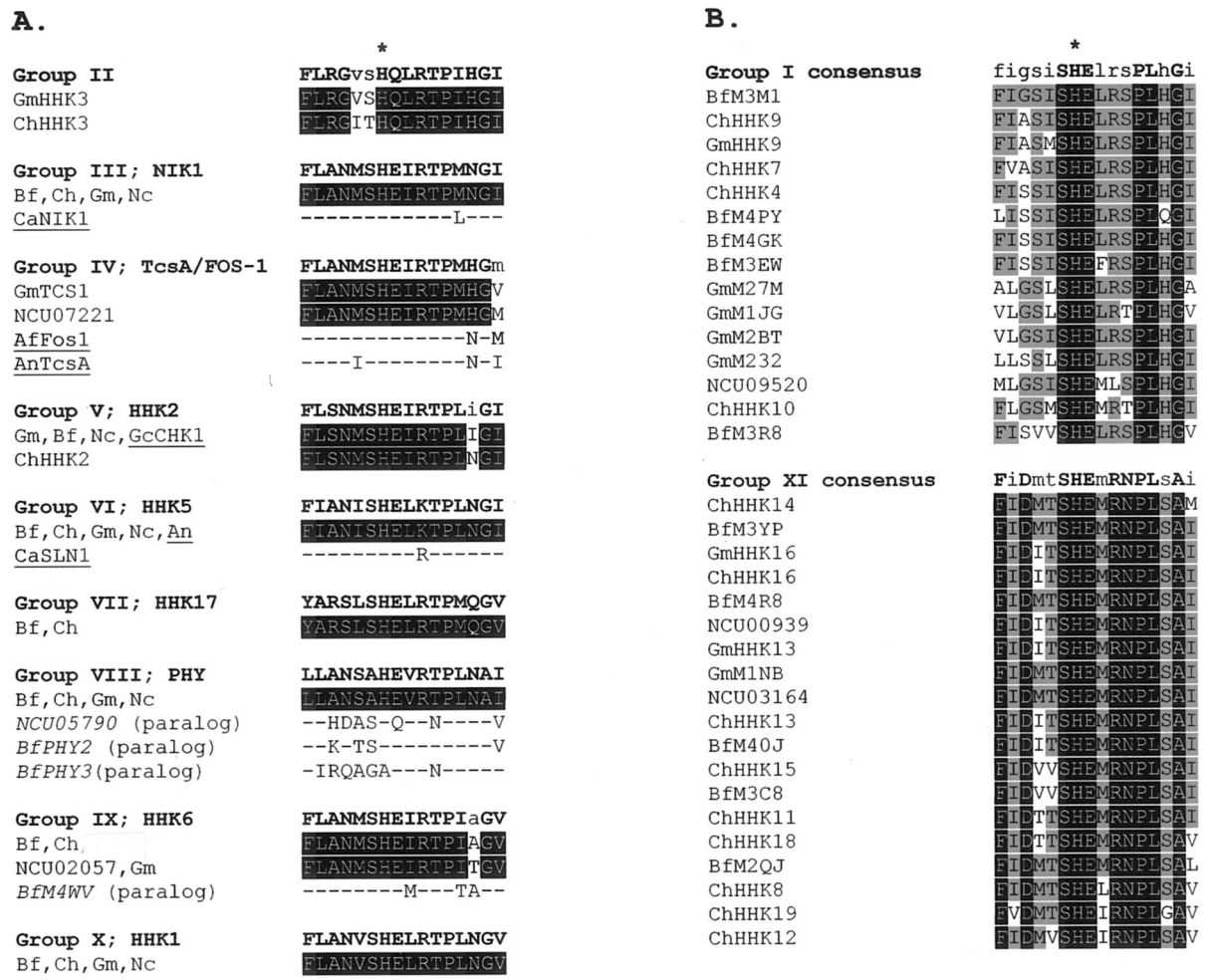

FIG. 3. Alignment of the H-box sequence containing the phosphoaccepting histidine. Subalignments of the H-box region (58) from the sequence alignment used for Fig. 2 are shown. For each group, the consensus sequences for *N. crassa* (Nc), *G. moniliformis* (Gm), *B. fuckeliana* (Bf), and *C. heterostrophu*s (Ch) are shown in bold at the top. (A) Conserved HK groups. Lowercase letters indicate amino acid residues that are not absolutely conserved among orthologs in the four euascomycetes considered here. Sequences from other ascomycetes (underlined) or paralogs (italicized) are represented by dashes for consensus (conserved) residues or the appropriate amino acid. (B) Divergent HK groups. All sequences are shown, even when two or more paralogs have identical H-box sequences. Absolutely conserved residues are shaded black, and residues conserved in at least 50% of all sequences are shaded gray. Asterisks indicate positions of conserved histidine residues.

for the most severe osmosensitivity and dicarboximide resistance phenotypes (17, 33).

Similar to the effects of mutations of *B. fuckeliana BfBOS-1* and *N. crassa NcNIK-1*/*OS-1*, disruption of the *C. heterostrophus ChNIK1* gene results in impaired growth relative to that of the wild type on solid medium containing 0.7 M NaCl (Catlett, unpublished), consistent with the idea that NIK1 generally functions in the osmotic response in euascomycetes. Unlike the euascomycete *NIK1* orthologs, however, *CaNIK1*/*COS1* has not been described as having an obvious role in osmotolerance, but it is required for normal serum-induced hyphal growth (2, 59). Thus, whether or not there is a universal role for *NIK1* in all ascomycetes remains unclear. HHK1 contains a long conserved N-terminal region with a partial protein kinase domain (PFAM00069) and a GAF domain (PFAM01590) (Fig. 2B). Interestingly, this domain structure is very similar to those of *S. pombe* ScMAK2 and ScMAK3, suggesting that these HKs share a common ancestor with HHK1 (Fig. 4). CaHK1 also shares this domain structure (Fig. 4) but does not group with the euascomycete HHK1 orthologs (Fig. 2B). This result suggests that the use of an alignment and a tree based only on the HK and RR domains may not provide enough information to group together more evolutionarily distant HKs derived from a common ancestor.

To examine this relationship further, full-length CaHK1, SpMAK2, and SpMAK3 amino acid sequences were aligned with the euascomycete HHK1 amino acid sequences. Sequence identity was observed along the entire sequence length and was not confined to the HK and RR domains (data not shown). A phylogeny constructed by using parsimony indicated that SpMAK2 and SpMAK3, despite relatively low overall sequence identity to each other, likely diverged from a common ancestor after the divergence of the archiascomycetes (Fig. 4). A *CaHK1* deletion causes flocculation of hyphae and reduced virulence, perhaps as a result of the misregulated expression of cell surface molecules (14, 59). SpMAK2 and SpMAK3 regu-

TABLE 2. Hybrid HKs organized by group[a]

Group	Founder(s)	Function	HK in:			
			Nc	Ch	Gm	Bf
I			*NCU09520*[b]	*ChHHK4*[c]	*GmM27M*	*BfM3EW*[d]
				ChHHK7	*GmM2BT*	*BfM3R8*[d]
				ChHHK9	**GmHHK9**	
				ChHHK10	*GmM1JG*	*BfM4GK*[c]
					GmM232_1	*BfM4PY*[d]
						BfM3M1[c]
II				**ChHHK3**	**GmHHK3**	
III	*NcNIK1*/*OS-1*	Putative osmosensor	**NCU02815.1**	**ChNIK1**[c]	**GmNIK1**	**BfBOS1**
IV	*AfFos1*, *AnTcsA*	Conidiation	**NCU07221.1**		**GmTCS1**	
V	*GcCHK1*, *SpMAK1*		**NCU01833.1**	**ChHHK2**	**GmHHK2**	**BfHHK2**[d]
VI	*ScSLN1*, *AnTcsB*	Transmembrane	**NCU04615.1**	**ChHHK5**	**GmHHK5**	**BfHHK5**[c]
VII				**ChHHK17**		**BfHHK17**
VIII		Phytochrome	**NCU04834.1**	**ChPHY1**[c]	**GmPHY1**	**BfPHY1**[c]
			NCU05790[b]			*BfPHY2*
						BfPHY3[d]
IX			**NCU02057.1**	**ChHHK6**	**GmHHK6**	**BfHHK6**[d]
						BfM4WV[d]
X	*SpMAK2*, *SpMAK3*		**NCU01823.1**	**ChHHK1**	**GmHHK1**	**BfHHK1**[d]
XI			**NCU00939.1**	*ChHHK13*[c]	**GmHHK13**	**BfM4R8**[d]
			NCU03164.1		**GmM1NB**	
				ChHHK16	**GmHHK16**[c]	
				ChHHK12		*BfM3C8*[c]
				ChHHK14		*BfM40J*[c]
				ChHHK15		*BfM3YP*[c]
				ChHHK8		*BfM2QJ*
				ChHHK18		
				ChHHK11		
				ChHHK19		

[a] Fungal HKs are grouped by species and class based on Fig. 2. Apparent orthologs are indicated in bold type; paralogs are indicated in italic type. Nc, *N. crassa*; Ch, *C. heterostrophus*; Gm, *G. moniliformis*; Bf, *B. fuckeliana.*
[b] Reannotated Whitehead Institute gene prediction.
[c] Annotated domains only (not the full peptide).
[d] Partial sequence (gaps).

late the oxidative stress response and the timing of mitosis via the RR SpMCS4 (5, 13, 38). The function of the homologous euascomycete *HHK1* genes is unknown.

The N-terminal domains of HHK2 orthologs contain two sets of PAS/PAC domains (Fig. 2B). These HKs are related to *Glomerella cingulata* GcCHK-1 (GenBank accession no. AAB19216), but the GcCHK-1 sequence is missing most of the N-terminal sequence found in other orthologs and thus likely is incomplete. The domain structure of HHK2 is similar to that of *S. pombe* SpMAK1.

HHK5 is related to CaSLN1, ScSLN1, and the recently reported AnTcsB. ScSLN1 is essential and acts as a sensor of the osmotic environment of the cell (31, 43). CaSLN1, unlike its *S. cerevisiae* ortholog, is not essential (37) but is involved in hyphal formation and virulence and appears to be essential for viability in combination with CaNIK1 (59). AnTcsB currently has no clearly defined role in *A. nidulans*, but AnTcsB can substitute for ScSLN1 in budding yeast (22).

HHK6 orthologs have a PAS motif at the extreme N terminus and multiple PAS/PAC motifs (Fig. 2B). *B. fuckeliana* contains a paralog of HHK6, BfM4WV, in addition to the ortholog BfHHK6 (Table 2 and Fig. 2A).

Phytochrome-related HKs were also found in each of the four filamentous fungal species and likely are present in most euascomycetes (Fig. 2A). *N. crassa* and *B. fuckeliana* contained two and three phytochromes, respectively. For each genome, one of the phytochromes (designated BcPHY1; NCU04834.1) appears to be orthologous to the other fungal phytochromes. Thus, the additional phytochrome-like HKs may be a result of duplication and divergence or, less likely, a result of selective loss from a common ancestor with multiple phytochromes.

Phytochromes, best characterized in plants, absorb and mediate responses to red and far-red light. Some phytochromes are less directly involved in responses to UV light (reviewed in reference 20). While plant phytochromes appear to be related to HKs, plant phytochromes lack many conserved residues essential for HK function (46) and may instead act as serine/threonine kinases (60). Nonphotosynthetic bacteria contain phytochromes thought to act as light-regulated HKs, functioning to protect cells from the harmful effects of light (12, 18). The fungal phytochromes described here appear to be similar to the bacterial phytochromes in the conserved amino-terminal chromophore-binding region (the GAF domain) (12). All of the fungal phytochromes examined here contain the conserved histidine residue required for biliverdin chromophore attachment and lack the cysteine residue used by plant phytochromes for bilin chromophore attachment (12, 18). Moreover, in contrast to the plant phytochromes, all of the fungal phytochromes examined here, except for BfPHY3, appear to contain the conserved residues necessary to function as light-regulated HKs.

Notably, Mooney and Yager predicted a phytochrome-like

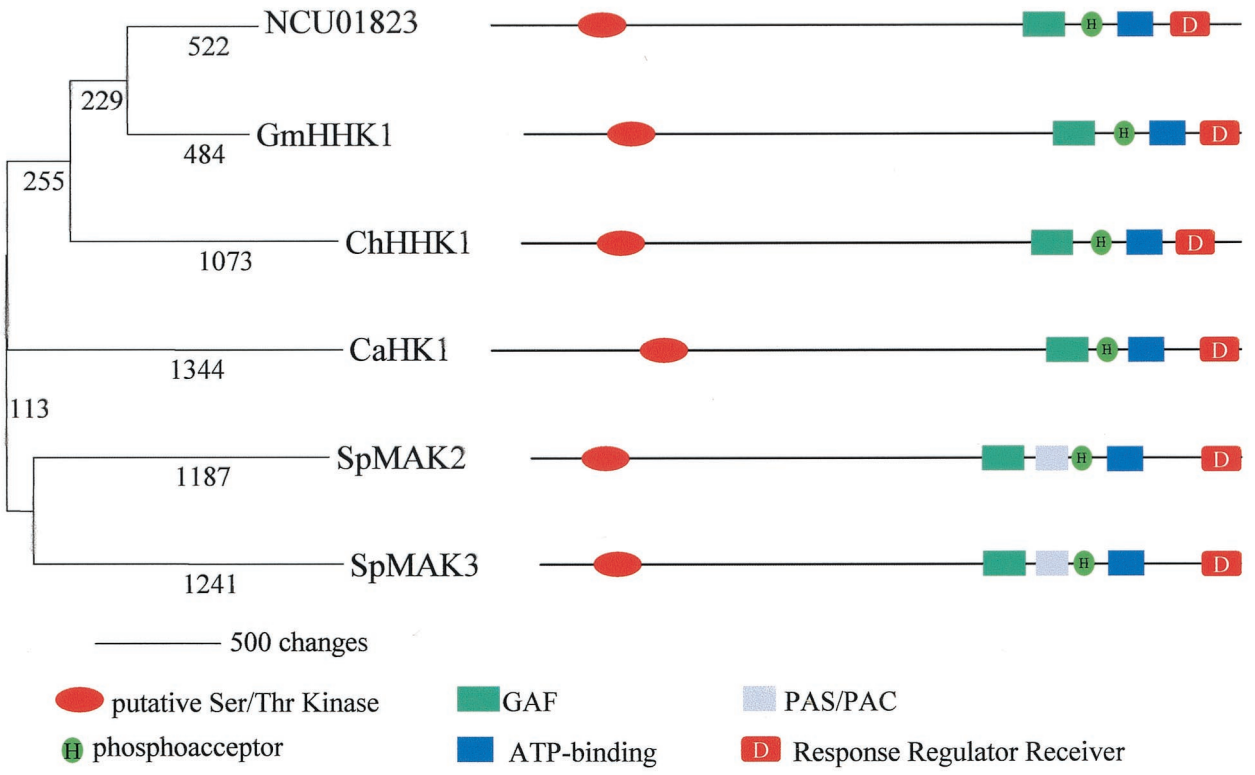

FIG. 4. Phylogram of HHK1 and related protein sequences. T-Coffee was used to align the NCU01823.1, GmHHK1, ChHHK1, CaHK1, SpMAK2, and SpMAK3 full-length peptide sequences. The phylogram was constructed by using parsimony. SpMAK2 and SpMAK3 are drawn as outgroups on the basis of bootstrap analysis. Numbers indicate branch lengths. BcHHK1 was omitted from the analysis because of missing sequence information. A scaled cartoon of the domain structure is shown to the right of each sequence.

photoreceptor in *A. nidulans* on the basis of the observation that conidiation is induced by red light and that this induction is reversed by far-red light (36). Similarly, light-regulated activity with a phytochrome-like action spectrum has been reported for *B. fuckeliana* (reviewed in reference 25). Many other aspects of fungal biology are affected by light, including entrainment of circadian rhythms and sexual development (25, 30), suggesting possible roles for these fungal phytochromes. No obvious growth or conidiation defects were observed in *C. heterostrophus ChPHY1* disruptants grown on solid medium under standard growth conditions (22°C, 12 h of UV light, 12 h of dark) (Catlett, unpublished). Lesions produced by the *phy1* mutants were indistinguishable from those caused by the wild type, suggesting that *ChPHY1* has no role in virulence on corn. Dissection of the biological roles of these fungal phytochromes may be complicated because of interactions of multiple photoreceptors, in addition to phytochromes, acting at different wavelengths (25).

HK groups not present in all species. In several instances, a group of HKs appears to be present in some but not all of the species examined. In each instance, the sequences were conserved in the N terminus as well as in the C-terminal HK and RR regions. For example, *ChHHK17* and *BfHHK17* are present in *C. heterostrophus* and *B. fuckeliana* but not in any of the other fungal genomes. Likewise, *ChHHK3* and *GmHHK3* are present only in *C. heterostrophus* and *G. moniliformis*. The functional significance of this distribution is unclear, as the functions of these genes are unknown. Deletion of *ChHHK3* resulted in no discernible effects on growth, conidiation, or sexual development (Catlett, unpublished). No full-length similarities to nonfungal proteins that might provide clues to the function or origin of *HHK3* and/or *HHK17* were detected by BLAST searches of GenBank.

AnTcsA and *AfFOS-1* orthologs are not present in the TMRI/Syngenta *C. heterostrophus* or *B. fuckeliana* genome sequences but are present in those of *N. crassa* and *G. moniliformis*. Disruption of the *AnTcsA* and *AfFOS-1* genes in *A. nidulans* and *A. fumigatus* is reported to affect conidiation (8, 44). Deletion of *AnTcsA* blocks the formation of conidia and conidiogenic phialides under normal growth conditions. However, conidiophore stalks and metulae form normally, suggesting that *AnTcsA* is not involved in the initiation of conidiation (8). Deletion of *AfFOS-1* is reported to result in a delay of conidiation in liquid medium (44).

Perhaps the distribution of *TcsA* and *FOS-1* HHK orthologs across species can be explained by differences in how conidia develop. Both *Aspergillus* and *Gibberella* species produce conidia enteroblastically from specialized flask-like cells called phialides; the outer layer of the conidiogenous cell wall is ruptured and does not contribute to conidium cell wall formation. In contrast, *Botryotinia* and *Cochliobolus* species conidia form holoblastically; all layers of the conidiogenous cell wall are involved in the formation of the conidium cell wall. In *Neurospora* species, macroconidia also form holoblastically;

TABLE 3. RR genes[a]

Gene[c]	Feature	RR gene in:			
		Nc	Ch	Gm	Bf
SSK1		NCU01895.1	*ChSSK1*	*GmSSK1*	*BfSSK1*
SKN7	HSF-type DNA-binding domain	NCU02413.1	*ChSKN7*	*GmSKN7*	*BfSKN7*
RIM15[b]	Protein kinase domain	NCU07378.1	*ChRIM15*	*GmRIM15*	*BfRIM15*
Other[d]			*ChREC1*	*GmREC1*	

[a] Nc, *N. crassa*; Ch, *C. heterostrophus*; Gm, *G. moniliformis*; Bf, *B. fuckeliana*. *ChREC1* and *GmREC1* do not appear to be orthologs.
[b] The *N. crassa*, *C. heterostrophus*, *G. moniliformis*, and *B. fuckeliana RIM15* ortholog sequences have an RR domain; the *SpCEK1* sequence does not.
[c] Gene names follow *S. cerevisiae* convention.
[d] Not found in *S. cerevisiae*.

however, microconidia are reported to form from phialides (reviewed in references 32 and 52). It will be interesting to examine strains carrying a disruption of the *N. crassa NcFOS-1* ortholog (NCU07221.1) for both macro- and microconidiation-related phenotypes. The probable absence of a *GzTCS1* ortholog in *G. zeae*, however, confounds this hypothesis.

Expanded families. Two groups of fungal HKs, I and XI (Fig. 2A and Table 2), identified through the phylogenetic analysis are highly divergent within the group (Fig. 3) and have been expanded in the euascomycete pathogens relative to *N. crassa*. While within each group, members shared similarities over most of the length of the protein, no full-length homologies were observed with nonfungal proteins that might provide information about the functional origins of these proteins.

Group I members contain a GAF-related domain. Few bootstrap-supported branches were observed within this group. While *N. crassa* contains only one member, NCU09520, *C. heterostrophus* contains four members and *B. fuckeliana* and *G. moniliformis* contain five members each (Fig. 2A and Table 2).

Group XI members generally contain a PAS/PAC domain adjacent to the HK domain (Fig. 2B). Most members contain a GAF-related domain, and a few (FvM1NB, NCU03164.1, and ChHHK14) contain additional PAS/PAC sequences. Group XI contains two members from *N. crassa*, NCU00939.1 and NCU03164.1. The predicted amino acid sequences for these two members share approximately 30% identity along most of the protein length. Each has a clear *G. moniliformis* ortholog (Fig. 2A and Table 2), reflecting that these two sequences likely diverged prior to the divergence of the *Sordariomycete* species. ChHHK13 and BfM4R8 may be orthologous to NCU00939.1, but these relationships are not well supported by bootstrap analysis. Group XI has undergone significant expansion in *C. heterostrophus*, with nine representatives, and in *B. fuckeliana*, with five. Interestingly, no sequences obviously related to this group were identified in a preliminary BLAST analysis of the TIGR *A. fumigatus* genome (http://www.tigr.org).

As no functions are known for any group I or XI HKs, it is difficult to guess the significance of the expansions. The duplication and divergence of these HKs may have allowed these species to evolve to adapt to different ecological niches, including the plant host. The repeat-induced point mutation phenomenon in *N. crassa* likely limited the expansion of its genome via duplication (51). Thus, to assess the possible significance for pathogenicity of the expansions, it will be useful to examine these groups of HKs in ascomycete saprobes without the same evolutionary limitations as *N. crassa*.

Transmembrane domains. The majority of bacterial HKs are transmembrane proteins with an extracellular sensor region. Thus, TMHMM (27), a transmembrane topology prediction method based on a hidden Markov model, was used to identify potential transmembrane domains in all fungal HK amino acid sequences. Only Sln1p and Hhk5p orthologs contained predicted transmembrane regions of any significance (Fig. 2B). In alignments of Hhk5p orthologs, the two predicted transmembrane domains in each ortholog aligned.

The lack of obvious transmembrane domains in any of these HKs, with the exception of Sln1p, Hhk5p, and TcsB, suggests that these signals may be internal, such as a redox-dependent signal. The presence of N-terminal functional domains, such as GAF and PAS/PAC domains, in many of these HKs is not inconsistent with this possibility. Alternatively, additional receptor proteins may receive extracellular signals and pass these signals on to intracellular HK proteins, as is necessary for signaling through the *Escherichia coli* chemotaxis HK CheA (reviewed in reference 28).

Downstream signaling. All hybrid HKs appear to function in multistep phosphorelays, in which the phosphate is transferred from the RR domain of the hybrid HK to a second histidine residue in an HPt (PFAM01627) domain and then to a second RR domain (Fig. 1) (16, 57). *S. cerevisiae* and *S. pombe* each encode one HPt domain protein, ScYpd1p and SpMpr1p (also called SpSpy1p), respectively (6, 38, 47). To gain further insights into two-component signaling downstream of the hybrid HKs in filamentous ascomycetes, we searched the fungal genome sequences for genes similar to *S. cerevisiae ScYPD1* and *S. pombe ScMPR1*. Each of the four filamentous ascomycete genomes examined (*N. crassa*, *B. fuckeliana*, *C. heterostrophus*, and *G. moniliformis*) appeared to encode only one HPt domain protein (Table 1). We named each euascomycete ortholog *HPT1* plus the two-letter species designation. The *N. crassa* HPt is NCU01489.1. *BfHPT1* was previously identified in an unannotated cDNA collection (Genoscope) (GenBank accession no. AL111646). Because HPt genes are relatively small, it is possible that some may remain to be found in the sequence gaps of these nearly complete genomes. Moreover, we may have failed to identify cryptic HPt sequences either as separate genes or within HK sequences.

We also searched for potential downstream RR genes. Each euascomycete genome examined contained apparent orthologs of the same RR genes as the yeasts (Table 3). The budding yeast HK, ScSln1p, signals through two RR proteins, ScSsk1p and ScSkn7p (29). ScSsk1p activates the *HOG1* MAP kinase pathway (42). ScSkn7p directly modulates transcription via a

heat shock factor (HSF)-type DNA-binding domain in ScSln1p-dependent and -independent pathways (29). *S. pombe* has similar proteins, SpMCS4 and SpPRR1, respectively (47). A third budding yeast protein, ScRim15p, involved in the expression of early meiotic genes (56), contains a C-terminal RR domain in addition to its N-terminal serine/threonine protein kinase domain. However, the functional significance of this RR domain has not been reported, and the apparent *S. pombe* ortholog, SpCEK1, lacks this domain (Table 3). All four filamentous ascomycete genomes examined contained *RIM15* homologs with RR genes. Both *C. heterostrophus* and *G. moniliformis* contained an additional potential RR gene. These genes, *ChREC1* (encoding 357 amino acids) and *GmREC1* (encoding 154 amino acids), do not appear to be orthologous to each other (data not shown). Moreover, phylogenetic analysis demonstrated no clear relationship of either putative amino acid sequence to the sequences of other fungal RRs or RR domains in the hybrid HKs (data not shown).

Given the expanded number of hybrid HKs in the euascomycetes compared to the yeasts (Table 1), it is striking that the number of downstream two-component signaling genes has not expanded similarly. In contrast, each of the five *E. coli* hybrid HKs appears to have a corresponding HPt domain, four as part of the hybrid HK and one as a separate protein (35). Furthermore, the *Arabidopsis* genome encodes five HPt proteins and one pseudo-HPt along with 22 possibly functional RRs and 9 pseudo-RRs (48). Despite a better correlation between the numbers of hybrid HKs, HPt domains, and RRs in bacteria and *Arabidopsis* compared to the euascomycetes, it should be noted that HPt domain proteins are promiscuous both in vivo and in vitro. For example, *Arabidopsis* HPt domain proteins function in yeasts (34). Moreover, the *Arabidopsis* HPt domain proteins may have overlapping functions (48).

Despite the significantly increased numbers of hybrid HK genes in the euascomycetes, the conservation of the HPt and RR genes with respect to that in the yeasts suggests that all hybrid HKs signal through the same downstream HPt and RR proteins. Possible advantages to such a signaling network include the potential to integrate multiple inputs into a single signaling pathway or to provide junction points for communication among several pathways (7). Notably, multiple-input HKs regulate *Bacillus subtilis* sporulation through the same RR (reviewed in reference 7). Multiple-input signals, such as nutrition, light, and temperature, regulate the developmental process of conidiation in *N. crassa* (52), perhaps utilizing unique HKs or other receptors for each signal.

Another possibility is that this small set of RRs may coordinate a different appropriate adaptive response for input signal to each HK by using unknown mechanisms to provide downstream specificity. These mechanisms could include developmentally regulated HK expression or compartmentalization of signaling components in different protein complexes and/or subcellular locations. Alternatively, some putative fungal hybrid HKs may not require these additional phosphorelay steps or even function as HKs.

In support of the hypothesis that multiple hybrid HKs function upstream of the same HPt and RRs, possible cross talk between *AnTcsA* regulation of conidiation and oxidative and osmostress pathways has been reported for *A. nidulans* (8). While *AnTcsA* deletion mutants have impaired conidiation, after extensive subculturing, colonies develop conidiating sectors. This suppression is blocked by oxidative stress and enhanced by osmotic stress. Similar cross talk or suppression could explain why deletion mutants of the *A. fumigatus AnTcsA* ortholog, *AfFOS-1*, are reported to have only a delay rather than a defect in conidiation (44).

Conclusions. Whole-genome analysis indicates that filamentous euascomycetes contain large numbers of HKs compared to yeasts. Many of these HKs are highly conserved among species, while others appear to have been subjected to duplication, divergence, and loss at some point in fungal evolution. Only a few of these HKs have been functionally characterized for any species. Thus, it remains unclear whether conserved HKs have similar functions in all ascomycetes. Moreover, future functional characterization of these HKs should elucidate whether the expansion of certain groups of HKs is indicative of functional redundancy and/or reflective of a need for fine-tuned sensing of the host environment by plant pathogens.

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